ANTIBIOFILM ACTIVITIES OF METAL MODIFIED KAOLINITE INCORPORATED PAINT AGAINST *MARINOMONAS COMMUNIS* AND *ALTEROMONAS* SP.

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To my beloved mother and father.

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ABSTRACT

Marine biofilm is a layer of microorganisms adhering on marine structures and has a major concern in marine industry. The detrimental effect of commercial antibiofouling coating to the environment increased the interest among researchers and industries in formulating new, effective and environmental friendly alternative antibiofouling coating. In this study, silver modified kaolinite and copper modified kaolinite incorporated paint (Ag-Kao-P; Cu-Kao-P) have been applied for the inhibition of biofilms of two marine bacterial strains, *Marinomonas communis* and *Alteromonas* sp.. The growth profiles of both bacteria were determined by monitoring the absorbance at OD_{600nm} and the viable cell count (CFU/mL). The antibiofilm efficacy of the Ag-Kao-P and Cu-Kao-P was tested via a homemade drip flow reactor system. The marine broth was allowed to flow continuously through flow reactor for 7 days at constant flow rate of 0.1 mL/min. The grown biofilm were observed under Atomic Force Microscopy (AFM). In a quantitative results, both Ag-Kao-P and Cu-Kao-P exhibit considerable antibiofilm activity against *M. communis* and *Alteromonas sp.*. Ag-Kao-P showed higher antibacterial activity against *M. communis* and *Alteromonas sp*. biofilm with 85% and 73% reduction, respectively. Conversely, only 76% biofilm reduction for *M. communis* and 60% to *Alteromonas* sp. for the Cu-Kao-P. Therefore, it can be concluded that Ag-Kao-P revealed the higher efficacy towards tested marine bacteria compared to Cu-Kao-P and can be suggested to be a future potential antibiofouling coating in marine industry.

ABSTRAK

Biofilem marin adalah lapisan mikroorganisma yang melekat pada struktur marin dan mempunyai kebimbangan utama dalam industri marin. Kesan memudaratkan lapisan antibiofouling komersil kepada alam sekitar telah meningkatkan minat dalam kalangan penyelidik dan industri bagi menformulasi lapisan antibiofouling alternatif yang baru, berkesan dan mesra alam sekitar. Dalam kajian ini, campuran cat dengan Argentum-Kaolinit dan Kuprum-Kaolinit (Ag-Kao-P, Cu-Kao-P) telah digunakan untuk mengkaji perencatan biofilem dua strain bakteria marin, iaitu *Marinomonas communis* dan *Alteromonas* sp.. Profil pertumbuhan kedua-dua bakteria telah ditentukan dengan memantau nilai penyerapan pada OD_{600nm} dan unit pembentukan koloni (CFU/ mL). Keberkesanan Ag-Kao-P dan Cu-Kao-P sebagai antibiofilem telah diuji dalam sistem reaktor aliran titisan yang dibina sendiri. Nutrien media marin telah dialirkan secara berterusan melalui reaktor aliran selama 7 hari pada kadar aliran malar 0.1 mL / min. Biofilem yang berkembang diperhatikan di bawah Mikroskop Daya Atom (AFM). Hasil secara kuantitatif, menunjukkan bahawa kedua-dua Ag-Kao-P dan Cu-Kao-P mempamerkan aktiviti antibiofilem yang tinggi terhadap *M. communis* dan *Alteromonas* sp.. Ag-Kao-P menunjukkan aktiviti antibakteria yang lebih tinggi terhadap biofilem *M. communis* dan *Alteromonas* sp. dengan pengurangan masing-masing sebanyak 85% dan 73%. Sebaliknya, hanya 76% pengurangan biofilem untuk *M. communis* dan 60% kepada *Alteromonas* sp. untuk Cu-Kao-P. Oleh itu, dapat disimpulkan bahawa Ag-Kao-P mempunyai keberkesanan yang lebih tinggi terhadap bakteria marin yang diuji berbanding Cu-Kao-P dan boleh dicadangkan untuk menjadi lapisan antibiofouling yang berpotensi pada masa depan dalam industri marin.

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LIST OF ABBREVIATIONS

$Al(OH)_3)$	Aluminium hydroxide
AF	Antifouling
AFM	Atomic force microscopy
CO_2	Carbon dioxide
CO	Carbon monoxide
CFU	Colony forming unit
Cu	copper
$(CuNO_3)_2\ 3H_2O$	Copper (II) nitrate trihydrate
Cu-Kao	Copper-kaolinite
Cu-Kao-P	Copper-kaolinite incorporated paint
DFR	Drip flow reactor
EPS	Extracellular polymeric substance
Au	Gold
IMO	International maritime organization
LPS	Lipopolysaccharides
MB	Marine broth
NO_x	Nitrogen oxides
OD	Optical density
PBS	Phosphate buffered saline
SEM	Scanning electron microscopy
SPC	Self-polishing copolymer
SiO_4	Silica

Ag	Silver
Ag^+	Silver ions
Ag-Kao-P	Silver kaolinite incorporated paint
$AgNO_3$	Silver nitrate
Ag-Kao	Silver-kaolinite
SO_x	Sulphur oxides
3D	Three dimensional
TBT	Tributyltin
TBT-SPC	Tributyltin self-polishing
2D	Two dimensional
UV	Ultra-violet

LIST OF SYMBOLS

Ra	Arithmetic average
cm	Centimetre
°C	Degree Celsius
g	Gram
hrs	Hours
mL	Mililiter
mm	Milimeter
mg	Milligram
M	Molar
N/m	Newton metre
ppm	Part per million
rpm	Rotation per minute
cm2	Square centimeter

CHAPTER 1

INTRODUCTION

1.0 Background

Biofilm formation has become a significant problem in various applications including medical, marine and industrial fields (Bixler and Bhushan, 2012). Biofilm is the predominant form of bacteria in the environment and exist when the microorganisms form a matrix of extracellular polymeric substance (EPS), responsible for their adhesion with each other and the surface (Kundukad *et al.*, 2016). The process of biofilm formation starts with reversible adhesion of planktonic bacterial cell onto a well-conditioned surface that will support their growth. This reversible adhesion will then turn into irreversible once they started to produce EPS. Progressively, they grow into microcolonies and form mature biofilms before finally, they disperse from the biofilm structure, spread and colonize other areas (Rasamiravaka *et al.*, 2015).

Over 99% of microorganisms have the ability to form biofilm on a wide range of surfaces (Sekhar *et al.*, 2009). The main function of EPS is to facilitate the initial attachment of cells to different substrates and protection against environmental stress and dehydration. According to Kristensen *et al.* (2008), the bacterial biofilm are resistant to harsh environment and antibiotics/biocides as well as improves the nutrient access and deliberates a more robust colonisation. It has been suggested that the production of EPS matrix on the biofilm, act as barrier enable the delay of initial penetration of antimicrobial agents (Mah and O'Toole, 2001).

The formation of undesirable marine biofilm on man-made structures such as ship hulls, pipelines, submerged structures and ocean research equipment has caused significant consequences (Hilbert *et al.*, 2003). In the shipping industry for example, the build-up of biofouling and biofilm led to an increase in surface roughness of ship hulls which will subsequently increase the hydrodynamic drag of the ship as it moves through the sea (Schultz *et al.*, 2011). This resulted in the increased of the fuel consumption increases (Callow and Callow, 2011). Consecutively, this event will also lead to the increase in costs within the shipping industry due to the increase in cost maintenance (Inbakandan *et al.*, 2013; Swain, 2010).

Besides causing the increment in costs, marine biofilm formation also causes detrimental effects towards the environment. The excessive emission of greenhouse gases (carbon monoxide (CO), carbon dioxide (CO_2), nitrogen oxides (NO_x) and sulphur oxides (SO_x)) (Eyring *et al.*, 2005) from the combustion of fuel by the marine vessel, had caused severe impact on global air quality. Not just that, biofilm and biofouling promotes the introduction of alien species to new ecosystems, overpopulating the area and has the possibility to act as predators to local species.

Over the years, a wide range of coating technology has been applied to ship hulls to battle against the marine organisms' attachment (Nurioglu and Esteves, 2015). Previously, biofouling has been controlled using toxic biocidal antifouling (AF) coating that is extensively exploited for marine industry (Salta *et al.*, 2013b). This AF coating work by releasing the toxic substances to the environment thus exposing the fouling organisms to the biocides (Ivče *et al.*, 2015). Historically, the self-polishing copolymer (SPC) technology containing tributyltin (TBT) has been the most effective AF coating (Chambers *et al.*, 2006; Korkut and Atlar, 2009). However, the use of TBT-based coating has been prohibited due their disastrous effects to non-target organisms and to the surrounding environment (Salta *et al.*, 2013b; Sonak *et al.*, 2009).

Therefore, the scientific communities have been focusing on studying and developing an effective and non-toxic coating (Statz *et al.*, 2006). The antimicrobial properties of metals have long been known and have been used since ancient time. According to Varkey (2010), metals such as copper and silver showed germicidal properties due to the oligodynamic effect in which can kill bacteria once introduced into the bacterial cells. Recent studies have utilized natural zeolites and clay minerals incorporated with metal ions to prepare new antimicrobial materials (Drelich *et al.*, 2011; Magana *et al.*, 2008). Yet, there is still lack of study that incorporated the use of this new antibacterial agent with the commercial paint that is available in the market.

1.2 Problem statement

Marine surfaces may act as reservoirs for microorganisms, causing the microbes to successfully attach onto the surface and developed biofilms. The presence of marine biofilm has a great impact on the marine industry, especially the shipping industry due to the negative impact on the performance, durability and maintenance of the ship (Peres *et al.*, 2014). Previously, antifouling coatings have been used in marine industry in order to combat the formation of biofilm (Salta *et al.*, 2013b). However, these biocidal coatings such as TBT had been identified to be toxic to aquatic animals as well as to the environment. A number of alternatives have been initially investigated for various marine applications with the intention to replace the use of TBT (Chambers *et al.*, 2006). However, their development is still in the laboratory scale while some other alternative is still considered as toxic to the environment. The antibacterial properties of silver-kaolinite (Ag-Kao) and copper-kaolinite (Cu-Kao) incorporated with commercial paint against marine bacteria has not yet being explored. Hence, this study might reveal the potential of Ag-Kao and Cu-Kao incorporated paint as a new antifouling coating that can be used in marine industry.

1.3 Objectives

The main objectives of this study are:

1. To design and test lab scale drip flow reactor that would simulate the continuous flow of marine environment.
2. To investigate the antibiofilm effects of silver kaolinite incorporated paint (Ag-Kao-P) and copper-kaolinite incorporated paint (Cu-Kao-P) against *Marinomonas communis* and *Alteromonas* sp. by calculating the viable cell by determining the colony forming unit (CFU).
3. To identify the morphological changes on *M. communis* and *Alteromonas* sp. cells following treatment with Ag-Kao-P and Cu-Kao-P using Atomic Force Microscopy (AFM).

1.4 Scope of study

The current study focuses on the determination of the antibacterial effects of Ag-Kao-Paint and Cu-Kao-Paint against *M. communis* and *Alteromonas* sp. which were cultivated in continuous flow environment. Besides that, this study also aims to identify the mechanism of action of Ag-Kao-P and Cu-Kao-P by observing the changes in bacterial cell morphology using AFM.

1.5 Significance of study

In marine industry, there is a real need for the continuous development of new non-toxic antifouling formulation. The determination of the potential and efficiency of Ag-Kao-P and Cu-Kao-P in preventing the growth of bacterial biofilm on the coated surface is the interest of this study. The outcome of this study will be a stepping stone in producing an environmental friendly antibiofouling paint which can be used primarily in marine industry.

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